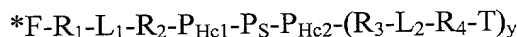


**WE CLAIM:**

1. A water-soluble peptidic substrate with the general formula:



wherein \*F is a detectable moiety with a molecular weight of less than 5 kD;

5

$R_1$ ,  $R_2$ ,  $R_3$ , and  $R_4$  are each, independently: a covalent bond or a covalent linkage consisting of a branched or unbranched, substituted or unsubstituted, saturated or unsaturated chain of 1-10 carbon atoms; 0-3 heteroatoms selected from the group consisting of oxygen, nitrogen, and sulfur; and further consisting of at least one linkage chosen from the group consisting of ether, ester, hydrazone, amide, thioether, thioester, thiourea, disulfide and sulfonamide linkages;

10

$L_1$  and  $L_2$  are each, independently: a branched or unbranched hydrophilic uncharged polymer selected from the group consisting of polyethylene glycol (PEG) and polysaccharides, and having a molecular weight of about 80 to about 4000 Daltons;

15

$P_{Hc1}$  is peptide with the general formula  $A_c(A_H)_nA_m$ ,

20

wherein  $A_c$  is selected from the group consisting of a covalent bond, ornithine, cysteine, homocysteine, cysteic acid, and lysine; each of  $A_H$  is, independently, a charged or uncharged hydrophilic amino acid selected from the group consisting of serine, threonine, lysine, arginine, histidine, aspartic acid, glutamic acid, and cysteic acid;

25

$n$  is an integer from 0 to 10;

$A_m$  is selected from the group consisting of a covalent bond and methionine;

30

$P_{Hc2}$  is a peptide with the general formula  $A_m(A_H)_nA_c$ ,

wherein  $A_c$ , if  $y$  is 1, is selected from the group consisting of a covalent bond, ornithine, cysteine, homocysteine, cysteic acid, and lysine; or, if  $y$  is 0, is a terminating group selected from the group consisting of alcohol moieties, amine moieties, ester moieties, ether moieties,

carboxylic acid moieties, amide moieties, and sulfonic acid moieties;

each of  $A_H$  is, independently, a charged or uncharged hydrophilic amino acid selected from the group consisting of serine, threonine, lysine, arginine, histidine, aspartic acid, glutamic acid, and cysteic acid;

$n$  is an integer from 0 to 10;

$A_m$  is selected from the group consisting of a covalent bond and methionine;

$P_S$  is a peptide from 5 to 25 amino acids in length;

$T$  is a terminating group selected from the group consisting of alcohol moieties, amine moieties, ester moieties, ether moieties, carboxylic acid moieties, amide moieties, sulfonic acid moieties, quencher moieties, and detectable moieties; and

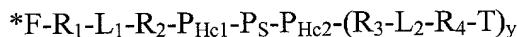
$y$  is 0 or 1.

2. The substrate of claim 1 wherein  $P_S$  comprises a known protein-kinase recognition sequence.
3. The substrate of claim 1 wherein  $P_S$  comprises a known protein-phosphatase recognition sequence.
4. The substrate of claim 1 wherein  $P_S$  comprises a known protease recognition sequence.
5. The substrate of claim 1 wherein  $P_S$  comprises a phosphorylated amino acid residue selected from the group consisting of phosphoserine, phosphothreonine, and phosphotyrosine.
6. The substrate of claim 1 wherein  $R_2$  is attached to the N-terminus of the peptidic portion of the molecule.
7. The substrate of claim 1 wherein  $R_2$  is attached to the C-terminus of the peptidic portion of the molecule.

8. The substrate of claim 1 wherein the non-peptidic portion of the molecule is uncharged.
9. The substrate of claim 8 wherein the peptidic portion of the molecule carries a net positive charge.
- 5 10. The substrate of claim 8 wherein the peptidic portion of the molecule carries a net negative charge.
11. The substrate of claim 8 wherein the peptidic portion of the molecule is carries no net charge.
12. The substrate of claim 1 wherein \*F is selected from the group consisting of a  
10 fluorescent moiety, a chromogenic moiety, and a chemiluminescent moiety.
13. The substrate of claim 1 wherein \*F is a fluorescent moiety.
14. The substrate of claim 13 wherein the fluorescent moiety is selected from the group consisting of BODIPY<sub>630/650</sub> X-SE, Texas Red X-SE, BODIPY TRX-SE, Cy-dyes, Lissamine, fluorescein, rhodamine, phycoerythrin, and coumarin.
- 15 15. The substrate of claim 1 wherein at least one of L<sub>1</sub> or L<sub>2</sub> is polyethylene glycol.
16. The substrate of claim 1 wherein at least one of L<sub>1</sub> or L<sub>2</sub> is a polysaccharide.
17. The substrate of claim 1 wherein at least one of L<sub>1</sub> or L<sub>2</sub> has a molecular weight of from about 100 to about 2000 Daltons.
18. The substrate of claim 1 wherein at least one of L<sub>1</sub> or L<sub>2</sub> has a molecular weight of  
20 from about 500 to about 1500 Daltons.
19. The substrate of claim 1 wherein at least one of L<sub>1</sub> or L<sub>2</sub> has a molecular weight of from about 800 to about 1000 Daltons.
20. The substrate of claim 1 wherein at least one of L<sub>1</sub> or L<sub>2</sub> is a polyethylene glycol having a molecular weight from about 230 to about 2000 Daltons.
- 25 21. The substrate of claim 1 wherein R<sub>2</sub> comprises an amide linkage.
22. The substrate of claim 1 wherein R<sub>2</sub> comprises a thiol linkage.
23. The substrate of claim 1 wherein for both P<sub>Hc1</sub> and P<sub>Hc2</sub>, A<sub>c</sub> is a covalent bond and n is 0.

24. The substrate of claim 1 wherein for at least one of  $P_{Hc1}$  and  $P_{Hc2}$ ,  $A_c$  comprises homocysteine.
25. The substrate of claim 1 wherein for at least one of  $P_{Hc1}$  and  $P_{Hc2}$ ,  $A_c$  comprises cysteine.
- 5 26. The substrate of claim 1 wherein  $P_{Hc1}$  has a different net charge than  $P_{Hc2}$ .
27. The substrate of claim 1 wherein  $P_{Hc1}$  has a negative net charge and  $P_{Hc2}$  has a positive net charge.
28. The substrate of claim 1 wherein  $P_{Hc1}$  has a positive net charge and  $P_{Hc2}$  has a negative net charge.
- 10 29. The substrate of claim 1 wherein  $P_S$  is from 5 to 10 amino acids in length.
30. The substrate of claim 1 wherein  $P_S$  comprises a random amino acid sequence.
31. The substrate of claim 1 wherein  $P_S$  comprises a weighted random amino acid sequence.
32. The substrate of claim 1 wherein  $P_S$  comprises a partially random amino acid  
15 sequence.
33. The substrate of claim 1 wherein  $P_S$  comprises a sequence selected from a known enzyme substrate.
34. The substrate of claim 1 wherein  $y$  is 0.
35. The substrate of claim 1 wherein  $y$  is 1.
- 20 36. The substrate of claim 1 wherein  $T$  is a terminating moiety selected from the group consisting of alcohol moieties, amine moieties, ester moieties, ether moieties, carboxylic acid moieties, amide moieties, and sulfonic acid moieties.
37. The substrate of claim 1 wherein  $T$  is a quencher moiety.
38. The substrate of claim 1 wherein  $T$  is a detectable moiety selected from the group  
25 consisting of a fluorescent moiety, a chromogenic moiety, and a chemiluminescent moiety.
39. The substrate of claim 1 wherein  $T$  is a fluorescent moiety different from \*F.

40. The substrate of claim 39 wherein T is selected from the group consisting of BODIPY<sub>630/650</sub> X-SE, Texas Red X-SE, BODIPY TRX-SE, Cy-dyes, lissamine, fluorescein, rhodamine, phycoerythrin, and coumarin.
41. A library consisting of a plurality of water-soluble peptidic substrates, wherein each peptidic substrate member of the library has the general formula:



wherein \*F is a detectable moiety with a molecular weight of less than 5 kD;

- 10 R<sub>1</sub>, R<sub>2</sub>, R<sub>3</sub>, and R<sub>4</sub> are each, independently: a covalent bond or a covalent linkage consisting of a branched or unbranched, substituted or unsubstituted, saturated or unsaturated chain of 1-10 carbon atoms; 0-3 heteroatoms selected from the group consisting of oxygen, nitrogen, and sulfur; and further consisting of at least one linkage chosen from the group consisting of ether, ester, hydrazone, amide, thioether, thioester, thiourea, disulfide and sulfonamide linkages;

- 15 L<sub>1</sub> and L<sub>2</sub> are each, independently: a branched or unbranched hydrophilic uncharged polymer selected from the group consisting of polyethylene glycol (PEG) and polysaccharides, and having a molecular weight of about 80 to about 4000 Daltons;

- 20 P<sub>Hc1</sub> is peptide with the general formula A<sub>c</sub>(A<sub>H</sub>)<sub>n</sub>A<sub>m</sub>,  
wherein A<sub>c</sub> is selected from the group consisting of a covalent bond, ornithine, cysteine, homocysteine, cysteic acid, and lysine;  
25 each of A<sub>H</sub> is, independently, a charged or uncharged hydrophilic amino acid selected from the group consisting of serine, threonine, lysine, arginine, histidine, aspartic acid, glutamic acid, and cysteic acid;  
n is an integer from 0 to 10;  
30 A<sub>m</sub> is selected from the group consisting of a covalent bond and methionine;

$P_{Hc2}$  is a peptide with the general formula  $A_m(A_H)_nA_c$ ,

wherein  $A_c$ , if  $y$  is 1, is selected from the group consisting of a covalent bond, ornithine, cysteine, homocysteine, cysteic acid, and lysine; or, if  $y$  is 0, is a terminating group selected from the group consisting of alcohol moieties, amine moieties, ester moieties, ether moieties, carboxylic acid moieties, amide moieties, and sulfonic acid moieties;

each of  $A_H$  is, independently, a charged or uncharged hydrophilic amino acid selected from the group consisting of serine, threonine, lysine, arginine, histidine, aspartic acid, glutamic acid, and cysteic acid;

$n$  is an integer from 0 to 10;

$A_m$  is selected from the group consisting of a covalent bond and methionine;

$P_S$  is a peptide from 5 to 25 amino acids in length;

$T$  is a terminating group selected from the group consisting of alcohol moieties, amine moieties, ester moieties, ether moieties, carboxylic acid moieties, amide moieties, sulfonic acid moieties, quencher moieties, and detectable moieties; and

$y$  is 0 or 1.

42. The library of claim 41 wherein, for each member of the library,  $P_S$  comprises a phosphorylated amino acid residue selected from the group consisting of phosphoserine, phosphothreonine, and phosphotyrosine.

43. The library of claim 41 wherein, for each member of the library,  $R_2$  is attached to the N-terminus of the peptidic portion of the molecule.

44. The library of claim 41 wherein, for each member of the library,  $R_2$  is attached to the C-terminus of the peptidic portion of the molecule.

45. The library of claim 41 wherein, for each member of the library, the non-peptidic portion of the molecule is uncharged.

46. The library of claim 45 wherein, for each member of the library, the peptidic portion of the molecule carries a net positive charge.
47. The library of claim 45 wherein, for each member of the library, the peptidic portion of the molecule carries a net negative charge.
- 5 48. The library of claim 45 wherein, for each member of the library, the peptidic portion of the molecule is carries no net charge.
49. The library of claim 41 wherein, for each member of the library, \*F is selected from the group consisting of a fluorescent moiety, a chromogenic moiety, and a chemiluminescent moiety.
- 10 50. The library of claim 41 wherein, for each member of the library, \*F is a fluorescent moiety.
51. The library of claim 50 wherein the fluorescent moiety is selected from the group consisting of BODIPY<sub>630/650</sub> X-SE, Texas Red X-SE, BODIPY TRX-SE, Cy-dyes, Lissamine, fluorescein, rhodamine, phycoerythrin, and coumarin.
- 15 52. The library of claim 41 wherein, for each member of the library, at least one of L<sub>1</sub> or L<sub>2</sub> is polyethylene glycol.
53. The library of claim 41 wherein, for each member of the library, at least one of L<sub>1</sub> or L<sub>2</sub> is a polysaccharide.
54. The library of claim 41 wherein, for each member of the library, at least one of L<sub>1</sub> or L<sub>2</sub> has a molecular weight of from about 100 to about 2000 Daltons.
- 20 55. The library of claim 41 wherein, for each member of the library, at least one of L<sub>1</sub> or L<sub>2</sub> has a molecular weight of from about 500 to about 1500 Daltons.
56. The library of claim 41 wherein, for each member of the library, at least one of L<sub>1</sub> or L<sub>2</sub> has a molecular weight of from about 800 to about 1000 Daltons.
- 25 57. The library of claim 41 wherein, for each member of the library, at least one of L<sub>1</sub> or L<sub>2</sub> is a polyethylene glycol having a molecular weight from about 230 to about 2000 Daltons.
58. The library of claim 41 wherein, for each member of the library, R<sub>2</sub> comprises an amide linkage.

59. The library of claim 41 wherein, for each member of the library,  $R_2$  comprises a thioether linkage.
60. The library of claim 41 wherein, for each member of the library, for both  $P_{Hc1}$  and  $P_{Hc2}$ ,  $A_c$  is a covalent bond and  $n$  is 0.
- 5 61. The library of claim 41 wherein, for each member of the library, for at least one of  $P_{Hc1}$  and  $P_{Hc2}$ ,  $A_c$  comprises homocysteine.
62. The library of claim 41 wherein, for each member of the library, for at least one of  $P_{Hc1}$  and  $P_{Hc2}$ ,  $A_c$  comprises cysteine.
63. The library of claim 41 wherein, for each member of the library, for at least one of  $P_{Hc1}$  and  $P_{Hc2}$ ,  $A_c$  comprises methionine.
- 10 64. The library of claim 41 wherein, for each member of the library,  $P_{Hc1}$  has a different net charge than  $P_{Hc2}$ .
65. The library of claim 41 wherein, for each member of the library,  $P_{Hc1}$  has a negative net charge and  $P_{Hc2}$  has a positive net charge.
- 15 66. The library of claim 41 wherein, for each member of the library,  $P_{Hc1}$  has a positive net charge and  $P_{Hc2}$  has a negative net charge.
67. The library of claim 41 wherein, for each member of the library,  $P_S$  is from 5 to 10 amino acids in length.
68. The library of claim 41 wherein, for each member of the library, wherein  $P_S$  comprises a random amino acid sequence.
- 20 69. The library of claim 41 wherein, for each member of the library,  $P_S$  comprises a weighted random amino acid sequence.
70. The library of claim 41 wherein, for each member of the library,  $P_S$  comprises a partially random amino acid sequence.
- 25 71. The library of claim 41 wherein, for each member of the library,  $y$  is 0.
72. The library of claim 41 wherein, for each member of the library,  $y$  is 1.
73. The library of claim 41 wherein, for each member of the library,  $T$  is a terminating moiety selected from the group consisting of alcohol moieties, amine moieties, ester



moieties, ether moieties, carboxylic acid moieties, amide moieties, and sulfonic acid moieties.

74. The library of claim 41 wherein, for each member of the library, T is a quencher moiety.

5 75. The library of claim 41 wherein, for each member of the library, T is a detectable moiety selected from the group consisting of a fluorescent moiety, a chromogenic moiety, and a chemiluminescent moiety.

76. The library of claim 41 wherein, for each member of the library, T is a fluorescent moiety different from \*F.

10 77. The library of claim 76 wherein, for each member of the library, T is selected from the group consisting of BODIPY<sub>630/650</sub> X-SE, Texas Red X-SE, BODIPY TRX-SE, Cy-dyes, lissamine, fluorescein, rhodamine, phycoerythrin, and coumarin.

78. A method of selecting peptidic substrates from the library of claim 41 for use in a protein-modifying enzyme assay, the method comprising:

15 (a) separating the members of the library which are soluble under suitable reaction conditions for the protein-modifying enzyme from those which are not soluble under suitable reaction conditions for the protein-modifying enzyme;

(b) combining the soluble members of the library obtained in (a) with the protein-modifying enzyme under suitable reaction conditions for the protein-modifying  
20 enzyme, thereby modifying some members of the library;

(c) separating the modified members of the library produced in (b) from the unmodified members of the library;

(d) determining the sequence of P<sub>s</sub> for the modified members of the library.

25 79. The method of claim 78 wherein the protein-modifying enzyme is a protein-kinase, and the modification of the modified members of the library is the phosphorylation of a serine, threonine, or tyrosine amino acid residue.

80. The method of claim 78 wherein at least a portion of the members of the peptidic substrate library contain a phosphorylated amino acid residue selected from the group consisting of phosphoserine, phosphothreonine, and phosphotyrosine, and wherein the  
30 protein-modifying enzyme is a protein-phosphatase, and the modification of the

modified members of the library is the dephosphorylation of a phosphoserine, phosphothreonine, or phosphotyrosine amino acid residue.

81. The method of claim 78 wherein the protein-modifying enzyme is a protease, and the modification of the modified members of the library is the cleavage of the peptidic  
5 portion of the modified members.
82. The method of claim 78 wherein the separation in (a) is by solvent phase partitioning between an organic solvent and an aqueous buffer suitable for use with the protein-modifying enzyme.
83. The method of claim 78 wherein the separation in (c) is by metal chelation  
10 chromatography.
84. The method of claim 83 wherein the metal chelation chromatography is carried out on a column containing a chelated cation selected from the group consisting of  $\text{Fe}^{+3}$  and  $\text{Ga}^{+3}$ .
85. The method of claim 78 wherein the separation in (c) is by chromatofocusing  
15 chromatography on an anion exchange column.
86. The method of claim 78 wherein the separation in (c) is by electrophoretic separation of the modified and unmodified members of the library.
87. The method of claim 78 wherein  $\text{R}_2$  is attached to the N-terminal end of the peptide portion of the peptidic substrates, and wherein the sequence determination in (d) is by  
20 C-terminal degradation of the peptidic portion of the modified members of the library.
88. The method of claim 78 wherein the sequence determination in (d) is by Edman degradation of the peptidic portion of the modified members of the library.
89. The method of claim 88 wherein  $\text{R}_2$  is attached to the C-terminal end of the peptide portion of the peptidic substrates.
- 25 90. The method of claim 88 wherein  $\text{R}_2$  is attached to the N-terminal end of the peptide portion of the peptidic substrates, and further comprising the step of cleaving the peptide portion of the peptidic substrates from the labeled hydrophilic polymer linker portion of the peptidic substrates.

91. The method of claim 90 wherein, for the members of the peptidic libraries,  $A_c$  in  $P_{Hc1}$  comprises methionine, and the cleavage is by cyanogen bromide cleavage of the substrates at the methionine residue.
92. A method of assaying a molecule of interest for its effect on a protein-kinase or  
5 protein-phosphatase reaction, the method comprising:
- (a) combining the molecule of interest, an enzyme selected from the group consisting of protein-kinases and protein-phosphatases, and one or more peptidic substrates of claim 1, wherein a  $P_S$  comprising a recognition sequence for the protein kinase is within one or more of the peptidic  
10 substrates, under conditions suitable for the activity of the enzyme;
  - (b) terminating the activity of the enzyme after a period of time;
  - (c) electrophoretically separating the phosphorylated peptidic substrate from the unphosphorylated peptidic substrate to produce a localized phosphorylated peptidic substrate fraction and unphosphorylated peptidic substrate fraction;
  - 15 (d) quantifying at least one of the separated fractions by detecting a detectable moiety on the peptidic substrate in the localized fraction, thereby determining the extent of conversion of the substrate by the enzyme during the period of time.
93. The method of claim 92, the method further comprising a step (e) comparing the extent  
20 of conversion of the substrate by the enzyme in step (d) with the extent of conversion by the enzyme when the enzyme is combined with the peptidic substrate under conditions suitable for the action of the enzyme for a substantially identical period of time in the absence of the molecule of interest.
94. The method of claim 92, the method further comprising a step (e) comparing the extent  
25 of conversion of the substrate by the enzyme in step (d) with the extent of conversion by the enzyme when the enzyme is combined with the peptidic substrate under conditions suitable for the action of the enzyme for a substantially identical period of time in the absence of the molecule of interest and in the presence of a molecule of known effect on the enzyme.
- 30 95. The method of claim 92 wherein the enzyme is a protein-kinase.

96. The method of claim 92 wherein the enzyme is a protein-phosphatase, and the peptidic substrates are phosphorylated in step (a).

97. The method of claim 92 wherein the period of time is in the range of 15 minutes to 2 hours.

5 98. The method of claim 92 wherein the period of time is in the range of 2 to 4 hours.

99. The method of claim 92 wherein the period of time is in the range of 4 to 8 hours.

100. The method of claim 92 wherein the period of time is in the range of 8 hours to 48 hours.

101. The method of claim 92 wherein the unphosphorylated peptidic substrate carries a  
10 net positive charge, and the phosphorylated peptidic substrate carries a net negative charge

102. The method of claim 92 wherein the unphosphorylated peptidic substrate carries no net charge, and the phosphorylated peptidic substrate carries a net negative charge.

103. The method of claim 92 wherein the unphosphorylated peptidic substrate carries a  
15 net positive charge, and the phosphorylated peptidic substrate carries no net charge.

104. The method of claim 92 wherein the unphosphorylated peptidic substrate carries a net negative charge, and the phosphorylated peptidic substrate carries a net negative charge.

105. The method of claim 92 wherein \*F is a fluorescent moiety, and the detecting in  
20 (c) is fluorometric detection.

106. A method of assaying a molecule of interest for its effect on a protease reaction, the method comprising:

(a) combining the molecule of interest, a protease, and one or more peptidic substrates of claim 1, wherein a P<sub>S</sub> comprising a recognition sequence for  
25 the protease is within one or more of the peptidic substrates, under conditions suitable for the activity of the protease

(b) terminating the activity of the protease after a period of time;

(c) electrophoretically separating the cleaved peptidic substrate from the uncleaved peptidic substrate to produce at least one localized cleaved peptidic substrate fraction and an uncleaved peptidic substrate fraction;

5 (d) quantifying at least one of the separated fractions by detecting a detectable moiety on the peptidic substrate in the localized fraction, thereby determining the extent of conversion of the substrate by the protease during the period of time.

107. The method of claim 106, the method further comprising a step (e) comparing the extent of conversion of the substrate by the protease in step (d) with the extent of  
10 conversion by the protease when the protease is combined with the peptidic substrate under conditions suitable for the action of the protease for a substantially identical period of time in the absence of the molecule of interest.

108. The method of claim 106, the method further comprising a step (e) comparing the extent of conversion of the substrate by the protease in step (d) with the extent of  
15 conversion by the protease when the protease is combined with the peptidic substrate under conditions suitable for the action of the protease for a substantially identical period of time in the absence of the molecule of interest and in the presence of a molecule of known effect on the enzyme.

109. The method of claim 106 wherein the uncleaved peptidic substrate carries a net  
20 positive charge, and the portion of the cleaved peptidic substrate comprising \*F carries a net negative charge.

110. The method of claim 106 wherein the uncleaved peptidic substrate carries no net charge, and the portion of the cleaved peptidic substrate comprising \*F carries a net negative charge

25 111. The method of claim 106 wherein the uncleaved peptidic substrate carries a net negative charge, and the portion of the cleaved peptidic substrate comprising \*F carries a net positive charge.

112. The method of claim 106 wherein the uncleaved peptidic substrate carries no net  
30 charge, and the portion of the cleaved peptidic substrate comprising \*F carries a net positive charge.

113. The method of claim 106 wherein \*F is a fluorescent moiety, and the detecting in (c) is by fluorometric detection.